(Dyn1 I533A) to generate curvature from model membrane templates *in vitro*. Here using site-directed fluorescence labeling coupled to multiple, independent spectroscopic techniques and confocal imaging on Giant Unilamellar Vesicles (GUVs), we have resolved the role of VL1 in dynamin function. Contrary to current understanding, our characterization of the isolated Dyn1 PH domain in comparison to full-length dynamin reveals that the PH domain VL1 is primarily a sensor of membrane curvature that serves in partitioning dynamin to regions of high membrane curvature (i.e. the narrow membrane neck of an invaginated coated pit) in order to direct localized dynamin self-assembly. Similar to full-length dynamin, the isolated PH domain preferentially inserted into highly curved membrane bilayers. However, unlike the full-length molecule, the PH domain was unable to generate curvature from planar membrane templates on its own. Our studies further reveal that *in vivo* fission-incompetent Dyn1 I533A fails to distinguish membrane curvature *in vitro* and is defective in directing organized self-assembly on curved membrane templates. Our studies provide critical insights into the role of the PH domain VL1 in dynamin function and expand the repertoire of PH domain functionality in protein-protein and protein-membrane interactions.

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High-Resolution 3D Reconstruction of a Dynamin Mutant, K44A, in its Super-Constricted State

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Dynamin assembles around the necks of budding vesicles during clathrin-mediated endocytosis and membrane trafficking in the cell. Upon GTP hydrolysis, dynamin constricts the neck, leading to membrane fission. We have previously shown that the presence of GTP drives dimerization of opposing GTPase domains in assembled dynamin polymers. This evokes a power stroke in the structure, which mediates membrane fission. Exactly how fission occurs in still unclear. Failure of dynamin to hydrolyze GTP results in block of endocytosis in the cells. To further elucidate the role of GTP hydrolysis during dynamin-mediated fission, we examined a GTP hydrolysis mutant, K44A. Here, we show that in the presence of GTP, K44A tubulates liposomes and constricts the underlying lipid to an extent far exceeding what was previously observed with other dynamin mutants. Sedimentation assays further show that, unlike wt dynamin, K44A-decoration of the lipid tube persists in the presence of GTP even after 30 minutes of incubation. In addition, we solved the 3D structure of K44A super-constricted tubes using cryo-electron microcopy and images processing methods. The 3D map revealed an inner diameter of ~4 nm, a diameter predicted to be sufficient for spontaneous membrane fission. We have further docked the crystal structures of individual dynamin domains into our 3D map and the best fit for the GTPase domain is in the GTP state and not the GDP/AlFx transition state. This suggests that GTP binding, and not GTP hydrolysis, drives constriction of the dynamin polymer into a super-constricted pre-fission state. Following constriction, the GTP-hydrolysis-driven power-stroke may then ultimately lead to disassembly of the polymer, resulting in membrane fission.

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Mutations Associated with Centronuclear Myopathy (CNM) Enhance the Size and Stability of Dynamin 2 Clusters in Cells

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Dynamin 2 (Dyn2), a ~ 100 kDa, GTPase that self-assembles into rings around the necks of budding vesicles, has been implicated in generating force for endocytic vesicle scission from the plasma membrane. Self-association of Dyn2 at the site of membrane invagination, and subsequent catalysis of membrane scission, is tightly coupled to GTP binding and hydrolysis. Mutations that affect the stability of Dyn2 polymers have been linked to autosomal dominant forms of CNM, a congenital disorder characterized by muscle weakness and wasting. In vitro biochemical analysis revealed that CNM-causing Dyn2 mutants express enhanced GTPase activity relative to wild-type Dyn2, and form polymers that are more resistant to GTPase-dependent disassembly. We compared the physical properties of assembled wild-type and CNM-linked mutant forms of Dyn2 in the cytosol and plasma membrane of living cells using fluorescence fluctuation spectroscopy (FFS). Our data indicate that unassembled wild-type Dyn2 is a tetramer in the cytosol, whereas CNM-associated R369W and A618T mutations induce the formation of higher order cytosolic oligomers (10- and 22-mers, respectively). FFS coupled with total internal reflection microscopy established that mutant forms of Dyn2 assemble into larger, more stable clathrin-containing structures on the plasma membrane than wild-type Dyn2. Based on these observations, a model to explain the defects in membrane trafficking in CNM muscle cells was developed.

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Characterizing MHC-I Delivery to Cell Plasma Membrane: A Spatiotemporal Study

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In our work, we aim at characterizing the delivery of Class I Major Histocompatibility Complex (MHC-I) molecules to the plasma membrane. Three aspects of MHC-I dynamics were investigated: delivery rate, position of delivery events and synthesis and delivery of new molecules.
Using Total Internal Reflection Fluorescence Microscopy (TIRFM) in combination with image analysis, we quantify the delivery rate of MHC-I to the plasma membrane. Furthermore, we demonstrate that inhibition of Dynamin reduces MHC-I delivery rate, a result that leads to the important conclusion that MHC-I molecules are primarily trafficked by Clathrin-coated vesicles. We find that MHC-I molecules are transported to specific, non-random locations on the plasma membrane, with possible implication on the interaction of MHC-I with their receptors on T lymphocyte cell membranes. Finally, comparing experimental Fluorescence Recovery After Photobleaching (FRAP) data with simulated recovery, we show that fluorescence recovery cannot be ascribed to lateral diffusion alone, and has an additional component originating in the delivery of newly synthesized MHC-I to the plasma membrane. These results may shed new light on the rates of synthesis and delivery of MHC-I molecules.

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Protein Structure Effects on Membrane Bending by Protein-Protein Crowding

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Two major mechanisms of cellular membrane bending during processes such as clathrin-mediated endocytosis have been previously proposed: bending by curved protein scaffolds such as a clathrin coat, and bending by insertion of wedge-like amphipathic helices into the membrane by adaptor proteins such as epsin1. Recently we have reported a third general membrane bending mechanism; bending by protein-protein crowding, where pressure generated by densely bound proteins drives membrane bending(1). Several endocytic adaptor proteins consist of a folded N-terminal membrane binding domain, and an unfolded C-terminal domain that binds clathrin and other proteins. Due to their lack of structure, the unfolded protein domains have much larger hydrodynamic radii than folded protein domains, potentially increasing the effects of their crowding compared to proteins of equal molecular weight. We have investigated the capability of these unfolded portions of adaptor proteins to bend membranes by binding them to giant unilamellar vesicles. using a Föster resonance energy tranfer based assay of protein density, developed in our previous studies, we find that the unstructured epsin C-terminus can bend model membranes at substantially lower densities than the structured epsin N-terminal homology domain, which has traditionally been thought to drive bending. These findings suggest that concentrating unfolded domains of adaptor proteins at endocytic sites may have a previously unappreciated role in promoting membrane bending. We also find that the addition of clathrin can locally increase the concentration of epsin1 on the membrane surface. Our ongoing experiments are investigating how clathrin and adaptor proteins work together to curve membrane surfaces.
(1) Stachowiak, J.C. *et al*., *Nat. Cell Biol*. **14**, 944, (2012).

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Polarized-Tirf-Based Monitoring of Sub-Resolution Membrane Curvature Dynamics during Clathrin-Mediated Endocytosis

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Our understanding of clathrin-mediated endocytosis is derived from analysis of live-cell protein recruitment kinetics in combination with static ultrastructure images of coated pit progression. These analyses however, cannot directly correlate membrane curvature dynamics with the arrival and activities of the endocytic machinery. Polarized Total Internal Reflection (pol-TIR) fluorescence microscopy can visualize membrane topology in cells labeled with lipophilic fluorophores whose dipoles align relative to the plasma membrane. We describe a new approach for creating s-pol and p-pol TIR fields in a commercial microscope utilizing a 2-dimensional scan head to position polarized